Biochimica et Biophysica Acta, 981 (1989) 173–177
Elsevier

BBA 74427

Changes in the proteolytic activity of human erythrocyte membrane during red cell aging

Maria Gaczyńska

Laboratory of Biophysics of Development and Aging, Department of Biophysics, University of Łódź, Łódź (Poland)

(Received 7 November 1988)
(Revised manuscript received 23 January 1989)

Key words: Aging; Erythrocyte membrane; Proteolysis

The action of endogenous membrane proteinases in membranes isolated from human red cells of various ages was assayed by three groups of methods: (1) determination of the amount of protein fragments released to the acid-soluble fraction; (2) monitoring of changes in ESR spectra of maleimide spin-labeled erythrocyte membranes; (3) electrophoretic methods: a two-dimensional analysis and analysis of the activity inside SDS-PAGE gels. For all the methods the effects of proteinase action were highest in ghosts isolated from the erythrocytes of middle age.

Introduction

Human erythrocyte ghosts exhibit the activity of several proteolytic enzymes, including thiol, serine and metalloproteinases [1–4]. Both endogenous (membrane proteins) and exogenous (casein, haemoglobin and albumin, under some conditions) substrates are degraded by the proteinases [1–4]. During red cell life, proteolytic enzymes (both cytosolic and membrane-associated) take part in maturation and senescence: they degrade superfluous proteins, produce families of structurally related proteins (syndeins, possibly sugar transporter) and are probably responsible for the production and exposition of the 'senescent cell antigen', a proteolytic fragment of band 3 protein [3–6]. Changes in the rate of self-digestion (digestion of membrane proteins during ghosts incubation) have been reported for various pathologies [7–9]. Taking into account the role of membrane proteinases in aging processes, studying the effects of the enzymes' action in ghosts isolated from red cells of various ages seems to be promising. The problem is interesting, given that the increasing effects of endogenous proteolysis during cell aging are apparently accompanied by decreasing activity of at least some proteinases [10–12].

Materials and Methods

Blood was collected from healthy donors and anticoagulated with heparin. Erythrocyte fractions of various ages (density) were obtained according to the method of Murphy [13]. The stratified cell column was divided into five equal-size fractions, of which the top one (youngest cells), the middle one (cells of middle age) and the bottom one (oldest cells) were further analysed. Erythrocytes were washed five times with PBS and the 'buffy coat' was removed. For better removal of leukocytes, cells were sedimented in 1.5% Dextran in PBS and passed through a cellulose (Serva) column. White ghosts were prepared by the slightly modified method of Dodge et al. and suspended in Buffer A [14,15]. Protein content was estimated by the modified method of Lowry et al. [16,17]. All incubations were performed at 37°C. The effects of proteinase action were assayed in membranes (in the presence of 100 μg/ml chloramphenicol) by the following methods.

Analysis of the protein content in the acid-soluble fraction

A ghost suspension (about 2 mg protein/ml) was mixed 1 : 1 (v/v) with 1% casein solution (BDH Chemicals, U.K.) (in Buffer A) and incubated for 0–24 h. Aliquots were taken every 2 h and prepared as in the method of Kunitz [18]. The protein content was mea-

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; ESR, electron spin resonance; Mal-6, *N*-(1-oxyl-2,2,6,6-tetramethylpiperid-4-yl)maleimide; Buffer A, 5 mM sodium phosphate buffer (pH 7.4); PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; SDS-PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulphate.

Correspondence: M. Gaczyńska, Laboratory of Biophysics of Development and Aging, Department of Biophysics, University of Łódź, Banacha 12–16, PL 90-237 Łódź, Poland.

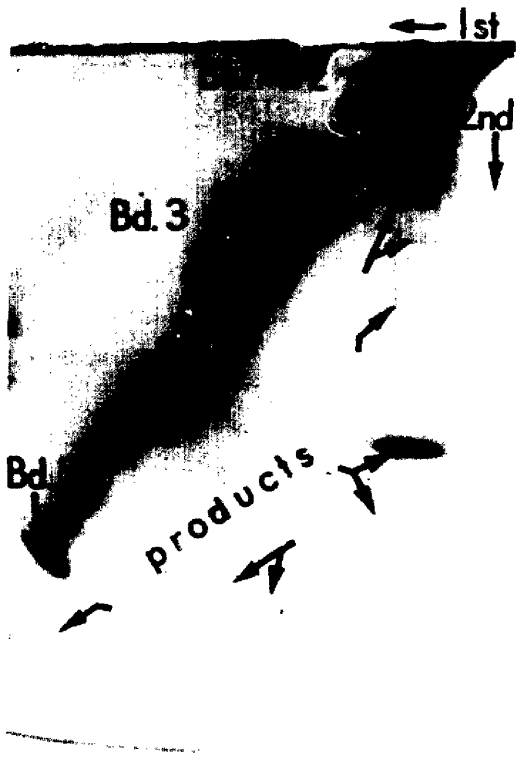

Fig. 1. Two-dimensional electrophoresis of erythrocyte membranes self-digested for 48 h at 37 °C. The first dimension (horizontal) - intact ghosts; the second dimension (vertical) - after gel (with membrane proteins inside) incubation. The main substrates and products are marked.

sured by the method of Lowry et al. [16]. Control samples added with buffer instead of casein solution were analysed in the same way. All results were calculated for unitary protein content in ghosts samples used. The increase in protein content was linear, so the regression equations could be assigned (regression coefficients were greater than 0.9). The slopes of the lines obtained for ghosts samples of various ages, but from one individual, were compared.

A method employing spin-labeled membranes

Ghost suspensions were spin-labeled with Mal-6 (Reanal, Hungary) as previously described [19] and ESR spectra were taken from 0 to 24 h, every 2 h, in a SE/X-28 spectrometer (Wroclaw Technical University, Poland). The ratio of low-field signal amplitudes of weakly (h_w) and strongly (h_s) immobilized spin-label residues increases during proteolysis (in general, proteolysis increases the participation of weakly immobilized Mal-6 residues in the protein molecule) [19]. The rate of h_w/h_s ratio changes could be the measure of the rate of proteolysis [19]. The increase in analysed samples was linear, the regression equations were calculated and the line coefficients were compared, as above.

Electrophoretic methods

(1) A two-dimensional electrophoretic technique was employed for self-digestion studies (Gaczyńska, submitted). Ghosts were dissolved in a medium containing SDS, DTT and glycerol in Tris-HCl buffer (pH 6.8) (37 °C, 30 min) and electrophoresed (the first dimension) in 10% or 7.5% polyacrylamide slab gels (stacking gel: 3%) in the presence of SDS (0.1%), according to Laemmli [20]. After 3–4 h of electrophoresis, the gels were incubated for 48 h in Buffer A and then gel strips (width of 8 mm) were submitted to SDS-PAGE (the second dimension) under reducing conditions [21]. The two-dimensional gels were stained with Coomassie brilliant blue R-250 [22]. On the electrophoretograms, the undigested proteins were placed on a diagonal, whereby the products of self-digestion 'inside the gel' were visible below the appropriate substrates (Fig. 1). Spectrin, band 3 protein, actin and products of their degradation were cut from the gels, the dye was eluted with 0.1 M NaOH/0.2% SDS and the relative protein amounts were analysed spectrophotometrically at 600 nm. The ratios of products/substrate amounts for ghosts of various ages, obtained from one individual, were compared.

(2) Proteolytic activity with respect to the exogenous substrates, casein and bovine serum albumin (Serva), was analysed in electrophoretograms [23]. Ghosts (0.1 mg of protein per cm^3 of gel) were electrophoresed in 10% slab gels, as described above. Electrophoretograms were cut into ten parts (Fig. 2), homogenized and incubated (48 h) in phosphate buffer containing 0.2 mg/ml of casein and 0.1 mg/ml of BSA. Samples of the substrate/protein solution were electrophoresed and stained as above. Casein, BSA and low-molecular weight

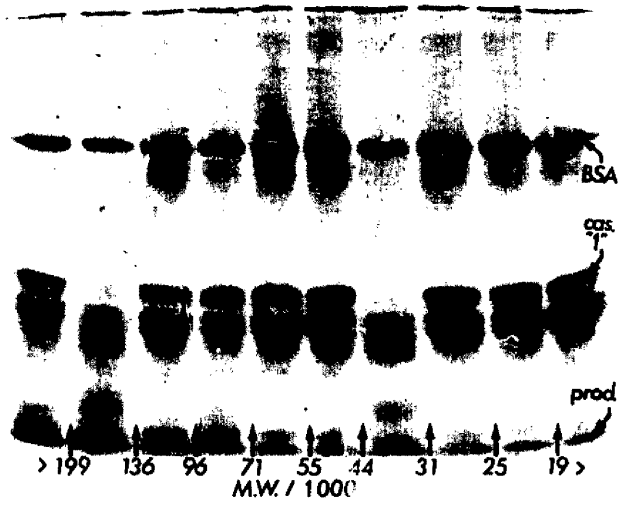

Fig. 2. Electrophoretograms of casein and BSA after incubation (48 h 37 °C) with ten gel fragments containing ghosts proteins after electrophoretic separation (10% gel). Relative molecular weights for cutting lines are described.

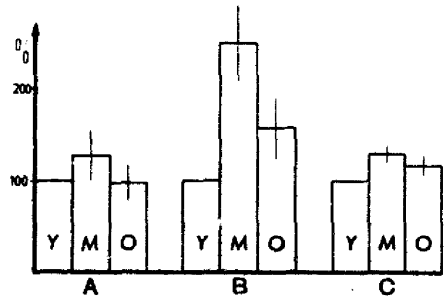

Fig. 3. Age-dependent changes in dynamics of casein digestion (A) and self-digestion (B, C) in ghosts. Bars represent mean ± S.E. A, relative values of line slopes for increase in protein content in acid-soluble fractions after casein digestion by ghosts; $n = 7$; difference M − O statistically significant for $P = 0.05$; B, as above, after self-digestion of ghosts; $n = 7$; difference M − Y significant for $P = 0.05$; C, relative values of line slopes for increase in h_w/h_s ratio in ESR spectra for self-digestion ghosts; $n = 11$; differences M − Y and M − O are significant for $P = 0.05$ and 0.01, respectively. Y, M, O represented the results for ghosts prepared from young, middle-age and old cells, respectively. Slopes for 'young' ghosts were assumed as 100%

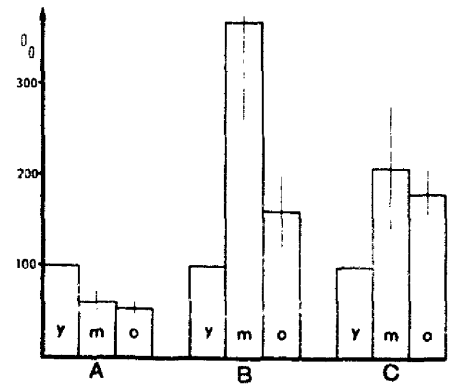

Fig. 4. Age-dependent changes in the products/substrate ratio for spectrin (A), actin (B) and band 3 protein (C) self-digested inside the gels by endogenous proteinases migrating near substrates (the two-dimensional technique). The bars show the mean ± S.E. Y, M, O are the results for ghosts prepared from young, middle-age and old cells, respectively. For spectrin differences Y − O, Y − M, M − O are statistically significant at $P = 0.01$, 0.01 and 0.001, respectively ($n = 8$). For band 3 ($n = 7$), Y − M difference $P = 0.01$. For actin ($n = 3$), M − Y difference $P = 0.01$. Ratios for 'young' samples (Y) were presented as 100%.

products (the end of the gel, see Fig. 2) were cut off from the gels, the dye was eluted and its concentration was measured as above. Then the ratio of products/substrates was calculated and compared.

For all results the significance of differences between 'young', 'middle' and 'old' samples was analysed by the paired T test.

Results

All the samples revealed proteolytic activity both against exogenous substrates and membrane proteins (self-digestion). For the analysis of protein release to acid-soluble fraction and for changes in ESR spectra, the slopes were taken as a measure of degradation dynamics. For all approaches employed, the dynamics were the highest for ghosts prepared from the cells of middle age (Fig. 3).

In Fig. 4, the relative ratios of proteolytic products to three appropriate substrate membrane proteins are presented. Proteinases analysed in this experiment migrated during electrophoresis in gel regions similar to those of substrates. Both for band 3 protein and actin the ratios (i.e., the effects of proteinase action) were the highest for the samples of middle age. However, for spectrin, an age-related decrease in degradation was observed. The two-dimensional technique was used instead of typical analysis of one-dimensional electropherograms because of the higher precision of quantitative analysis.

Electrophoretograms of human erythrocyte ghosts revealed proteolytic activity against casein and albumin for four gel regions (Table I). Intact ghosts did not digest BSA, whereas partially purified membrane proteinase did (Gaczyńska and Bartosz, manuscript in preparation). Not all 'active regions' were observed for all individuals: commonly, two or three 'activities' could be distinguished for one ghosts sample (Fig. 2). In the case of casein, for the quantitative analysis only, the first band was considered (the other bands could overlap with products of proteolysis; Fig. 2). The higher the products/substrate ratio, the higher was the relative activity of proteinase(s) of appropriate relative molecular weight. The tendency of changes in the activity was noted in Table I: for most gel regions, the 'activity' for ghosts prepared from erythrocytes of middle age was higher than for 'young' samples and approximately equal to the value for 'old' probes. In Fig. 5, the results for which the difference between 'young' and 'middle' age samples was statistically significant are shown.

TABLE I

Proteolytic activity in electrophoretograms (SDS-PAGE) of human erythrocyte ghosts

Y, M, O represent ghosts isolated from young, middle-age and old red cells, respectively; M_r, relative molecular weight.

Activity response with respect to			
casein		bovine serum albumin	
M_r ($\times 10^{-3}$)	age dependence	M_r ($\times 10^{-3}$)	age dependence
> 136	M = O > Y	> 199	–
71–36	M = O > Y	71–96	M > Y = O
44–58	–		
< 31	M = O > Y	< 31	M > Y = O

Discussion

One of the consequences of a membrane-isolation procedure is the activation of several endogenous membrane proteinases, which normally, in the intact cells, reveal only limited action [1,3,10,15]. Therefore, significant differences should be expected between the physiological proteolytic activity and the self-digestion which results in the destruction of membrane proteins pattern after several dozen hours of ghosts incubation [15]. In spite of the objections, analysis of the proteinase action in ghosts seems to be useful for comparative studies of membranes [8,9]. The two analytical approaches employed in this study, self-digestion and digestion of exogenous substrates, reflected the two main reasons of differences in proteolysis effects. Changes in the real activity of enzymes (changes in enzyme molecules) and in the amount of enzyme molecules would be better seen during cleavage of exogenous substrates than during self-digestion, for which variations in proteolytic susceptibility of natural substrates seem to be of at least the same importance.

In this study, the increase in the effects of proteinase action in ghosts prepared from erythrocytes of middle age was observed for all experiments (with one exception). For self-digestion the result seems to be easy to explain. The two opposite processes, described for red cells aging, i.e., the decrease in proteinase activity and the increase in proteolytic susceptibility of membrane proteins, could produce the observed effect [11,24]. For bovine membranes, a decrease in the rate of band 3 self-digestion and the decrease–increase–decrease pattern for spectrin were observed during erythrocytes storage (in vitro aging; Gaczyńska et al., Cytobios, in press). However, a similar increase in casein and albumin degradation, even under conditions of contaminations of self-digestion effects (activity in electrophoretograms, the Kunitz method after subtraction of results belongs to endogenous substrates digestion) required an additional explanation. Possibly, the activation of some proteinase(s), e.g., by the loss of a natural inhibitor or by changes in environmental conditions, takes place during aging. An exception from the rule observed, decreased degradation of spectrin remains unexplained. Proteinase(s) analysed in that experiment was probably only a part of the enzymes which would degrade spectrin. Spectrin, the main cytoskeleton protein would reveal the original pattern of age-dependent changes and not the same enzymes which would digest spectrin and integral band 3 protein [8,9]. The interpretation of the results obtained could be additionally complicated by the fact that not all proteinases were active under the conditions used (especially in SDS gels). All the phenomena observed require further study, especially with proteinases isolated from membranes.

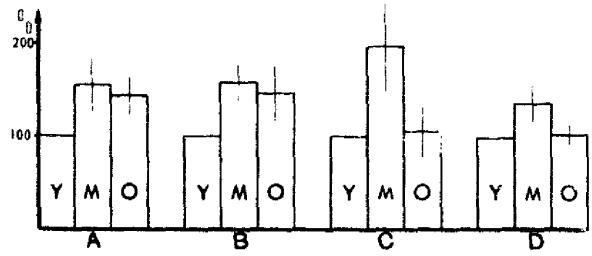

Fig. 5. Age-dependent changes in products/casein "1" (A, B) or albumin (C, d) ratio after incubation of ghosts electrophoretograms fragments with casein and BSA. (A) Gel fragments with proteins of relative molecular weights higher than 136000 ± 6000; differences Y – M and Y-O are statistically significant at $P = 0.02$ and 0.05, respectively. (B) Molecular weights from 71000 ± 4000 to 86000 ± 2000; for Y – M difference $P = 0.01$. (C) 71000 ± 4000 to 96000 ± 7000, for Y – M difference $P = 0.01$. (D) Relative molecular weights lower than 31000 ± 2000, for Y – M difference $P = 0.05$. The bars represent the mean ± S.E.; $n = 7$.

Acknowledgement

This work was performed within the framework of Project RP-II-11.4.8 of the Polish Ministry of Education.

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